A COMPARATIVE STUDY ON DIFFERENT DMEK GRAFT PREPARATION TECHNIQUES

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ABSTRACT

Purpose: To compare different Descemet Membrane Endothelial Keratoplasty (DMEK) graft preparation methods.

Methods: *Stripping from the trabecular meshwork (M1*) using epithelial spatula; *stripping by scoring the peripheral endothelium (M2)* using Sinskey hook; *stripping by punch method (M3*) using donor punch; *Submerged Hydro-Separation (M4*); and pneumatic *dissection method (M5*) were evaluated. Preparation time, costs, endothelial cell loss (ECL) post-preparation, cell death and morphology were compared. Hoechst/Ethidium/Calcien AM (HEC) staining and Zonula Occludens-1 (ZO-1) expression was analyzed. Statistical analysis was performed using Anova test and; Tukey as post-hoc test.

Results: 35 corneas (7 per group) were used. ECL represented as Mean(SD), in M1, M2, M3, M4 and M5 was 2.7(5.0), 3.0(7.4), 1.2(7.4), 3.3(7.3) and 4.1(7.1)% respectively not showing any difference between the groups ($p=0.96$). A significantly higher cell death ($p<0.05$) was observed in M4 and M5 compared to M1, M2 and M3. Graft preparation time was significantly shorter in M4 and M5 and longest in M3 ($p<0.05$). M3 was the most expensive preparation technique. Minimum pleomorphic cells were observed in M1, M2 and M3 whereas moderate pleomorphism was seen in M4 and M5. HEC staining showed high Ethidium positivity (dead cells) in M4 and M5 with minimum positivity in M1, M2 and M3. ZO-1 was expressed in all the conditions except the denuded areas.

Conclusion: Graft preparation using *Sinskey hook (M2)* and *donor punch (M3*) are reliable methods in terms of efficiency and quality with acceptable range of ECL. The preparation time and associated costs could be a limitation for M3.

KEYWORDS

DMEK; graft preparation; comparison

INTRODUCTION

Descemet Membrane Endothelial Keratoplasty (DMEK) has gained popularity for many surgeons to treat specific endothelial failure cases. DMEK offers early rehabilitation with optimal visual acuity [Melles et al. 2006; Kruse et al. 2011; Price et al. 2013]. Its minimally invasive nature and low immunologic rejection rate make this technique favourable for surgeons (Anshu et al. 2012). Even with these advantages, the technique still requires refinement in terms of graft preparation, delivery, unfolding, and positioning in the recipient eye (Terry 2012; Gorovoy 2014). Recently, the eye banks have been requested with pre-stripped DMEK tissues (Deng et al. 2015; Kobayashi et al. 2015) that aim to reduce the surgical efforts, time, and costs in the theatre. With such requests, the eye banks are challenged to find a standardized method in terms of feasibility of preparation, good graft quality, availability of the instruments and associated costs. Earlier, air bubble (Busin et al. 2010) and liquid bubble (Parekh et al. 2014) techniques to separate the stroma and Descemet Membrane (DM) has been described. Dapena et al. (Dapena et al. 2011) and Groeneveld et al. (Groeneveld et al. 2013) also suggested no-touch method for DMEK stripping. It was noted from the literature that the bubble and stripping techniques are uptaken easily and have been used for DMEK graft preparation. The following study therefore describes five methods that are slight modifications of the conventional bubble and stripping techniques and determine the effects of each technique on endothelial morphology and cell loss with an intention to highlight the most feasible option in terms of costs, quality and efficacy allowing eye bankers and surgeons to optimize the best choice for DMEK graft preparation.

MATERIALS AND METHODS

Ethical Statement

Human donor corneas [n=35] were collected from the Veneto Eye Bank Foundation, Mestre, Italy (FBOV) with written consent from the donors' next-of-kin for use in research. The study followed the tenets of 2013 Declaration of Helsinki.

Donor characteristics and tissue preservation

Donor characteristics were obtained from the FBOV database. All the corneas were preserved at 31^{o}C in tissue culture medium (TCM) that was composed of 2% newborn calf serum with MEM-Earle as a base medium along with 25 mM HEPES Buffer, 26 mM sodium bicarbonate, 1 mM pyruvate, 2 mM glutamine, 250 ng/mL amphotericin B, 100 IU/mL penicillin G, and 100 mg/mL streptomycin. TCM was prepared at FBOV with full regulatory compliance.

Endothelial evaluation

The endothelium of all the samples was stained using trypan blue (0.25%) for 20 seconds and washed with phosphate buffered saline (PBS). The corneas were placed in a sterile petri dish containing a hypotonic sucrose solution (to increase definition of cell borders) with the epithelium uppermost. To estimate endothelial cell density (ECD), the cells in five 1-mm^2 squares of a 10 X 10 mm reticule inserted in the eyepiece of an inverted microscope (Primovert; Zeiss, Jena, Germany) were counted manually at 100X magnification. The number of blue-stained (trypan blue positive cells - TBPCs) cells allowed percentage cell death to be determined. The ECD and cell death were counted both before and after DM stripping.

Alizarin red staining [n=3 per method]

After DMEK preparation, the endothelium was stained (immersed) with alizarin red S (Sigma Aldrich – A3757, Missouri, United States) for approximately 5 minutes, washed with PBS, and viewed at 100x magnification using an inverted microscope to observe the morphology (pleomorphism, polymegethism, and hexagonality) of the cells.

Hoechst, Ethidium homodimer and Calcein AM (HEC) staining to determine live/dead cells [n=2 per method]

The tissues after trypan blue staining were washed with PBS prior to HEC staining. 4 µL of Hoechst 33342 (H) (Thermo Fisher Scientific, Rochester, NY, USA), 4 µL of Ethidium Homodimer EthD-1 (E) and 2 µL of Calcein AM (C) (Live/Dead viability/cytotoxicity kit, Thermo Fisher Scientific, Rochester, NY, USA) was mixed in 1 mL of PBS and mixed well. 300 µL of the final solution was directly added on the endothelium of the DMEK tissue resting on the cornea and incubated at room temperature in the dark for 30 minutes. The DMEK lenticule was excised and placed on a slide with the endothelium uppermost. Four radial cuts were made to obtain a

flat mount of the tissue on the slide and covered with coverslips without the mounting medium. The endothelium was viewed at 100x magnification of Nikon Eclipse Ti-E (Nikon, Burgerweeshuispad, Amsterdam) using NIS Elements software (Nikon).

Expression of tight junctions using Zonula Occludens-1 (ZO-1) marker [n=2 per method]
The tissues were washed with PBS and fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 10 minutes. The cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. After blocking with 1% bovine serum albumin for 1 hour at RT, the tissues were incubated with 0.1% bovine serum albumin and primary antibody [anti-ZO-1, 1:200 (ZO1-1A12 Alexa Fluor 488 conjugate; Invitrogen, Carlsbad, California, USA)] for 3 hours at room temperature. After each step, the cells were washed thrice with 1X PBS. The tissue was flat mounted on the slide and fixed with a mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) to stain the nuclei. Cells were examined under Nikon Eclipse Ti-E (Nikon, Burgerweeshuispad, Amsterdam) microscope at 100X magnification and the images were captured with NIS Elements software (Nikon).

The study was divided into the following five groups, each with 7 corneas. M1 was performed by DB, M2 by VR, M3 by AR, M4 and M5 by MP. Each technician has prepared more than 50 tissues with the method they were assigned and therefore their level of experience was deemed suitable for this study.

Stripping from the trabecular meshwork – Method 1
A cornea was centered on a punch base using suction; however, it is not mandatory to use a suction base for this method. The tissue was stained with trypan blue for ease of visualization for 1 minute. The point of initiation (PoI) (starting point where the instrument is touched for incision) was 1mm away from the Trabecular Meshwork (TM) on the sclera. An epithelial spatula (Blink Medical Ltd., Birmingham, UK) was used to swipe the TM towards the cornea detaching the peripheral endothelium as observed in Figure 1A and 1B. The TM was excised completely exposing the periphery of the DMEK graft (Figure 1C). The DMEK was grasped at its periphery using a 120 mm straight pointed acute forceps (e.Janach, Como, Italy) and peeled using different peeling sites to release the DMEK tissue avoiding peripheral tears (Figure 1D). Note that trypan blue stain remains on the tissue keeping the tissue moist and there is no liquid (TCM) on the endothelium. The preparation method is shown in supplementary video 1.

Stripping by scoring the peripheral endothelium – Method 2
A cornea was centered on a punch base using suction; however, it is not necessary to use the suction base in this method. The PoI of this technique is at the periphery of the DMEK graft, just before the TM as seen in Figures 1E and 1F. An Intra Ocular Lens

(IOL) manipulator [Sinskey] hook with blunt tip (Beaver-Visitec International Ltd., Warwickshire, UK) was used to score the peripheral circumference on the endothelial side of the cornea (Figure 1G) detaching the periphery of the DMEK for further peeling. 120mm straight pointed acute forceps (e.Janach, Como, Italy) was used to peel the DMEK graft using a single peel (superior to inferior) method (Figure 1H). The cells were kept moist with a single drop of PBS and were not totally submerged in the liquid. In challenging cases where the tissue was tightly attached to the stroma, multiple quadrant method (where the edge of the DMEK tissue is grasped at different sites and peeled in parts obtaining a single graft) was used. Note that unlike method 1, the tissue can be stained to see the scored area if necessary. The method is shown in supplementary video 2.

Stripping by punch method – Method 3

A cornea was centered on a punch base using suction. Vacuum was created using a syringe and the cornea was secured on the base. The PoI of this technique can vary depending on the donor punch (8.25-10.00 mm diameter) that is selected. For this study, we used our standard protocol with a 9.5 mm donor trephine making it the PoI (Figure 1I). The endothelium was gently tapped with the trephine (Moria, Antony, France) to make a superficial cut (Figure 1J). Trypan blue was applied on the endothelium for about 20 seconds to determine the trephined zone. Excess peripheral endothelium was removed using 120mm straight pointed acute forceps (e.Janach, Como, Italy) and the tissue re-stained to help see the non-endothelial part. The endothelium was kept moist during the entire procedure using TCM to create a film of fluid on the top of the endothelium. The membrane was slightly lifted at the periphery using a cleavage hook and the procedure was continued along the entire circumference of the trephined area to ensure limited peripheral tearing while peeling (Figure 1K). The DMEK tissue was stripped with a longitudinal movement using a 3-quadrant method, ensuring no stress lines were generated, thus reducing cell death. Once the DM was stripped, leaving a peripheral hinge, it was laid back onto the stroma (Figure 1L) as previously described (Parekh et al. 2016). The method is shown in supplementary video 3.

For Methods 1, 2 and 3, the DM was not completely stripped but left attached by a peripheral hinge. The DM was laid back onto the stroma for morphological and molecular analysis as described above.

Submerged Hydro-Separation (SubHyS) Method – Method 4

In this technique, a cornea was submerged in a sterile petri dish half-filled with the TCM to keep the endothelium moist throughout the procedure. The tissue was held at the sclera with sterile forceps. A needle (25 gauge) connected to a 1 mL syringe was inserted beneath the TM (PoI) (Figure 1M and 1N) and advanced radially in the

posterior stroma or stroma–Descemet membrane interface until the bevel of the needle was completely inserted. TCM from the syringe was injected into the stroma or the DM-stroma interface with pressure enough to separate the DM and the stroma. A small, clear bubble was visible (Figure 1O) with the initiation of the process. The bubble was enlarged with an increased pressure to achieve an approximately 10-11 mm diameter bubble (Figure 1P). The liquid was removed by sucking the liquid back in the syringe (Parekh et al. 2014) and the tissue was used for further analysis. The method is shown in supplementary video 4.

Pneumatic dissection method – Method 5

In this technique, a cornea was placed in a sterile petri dish with endothelial side upwards. Using a 25G needle attached to a 1 mL syringe, the needle was advanced in a tangential direction from the limbus (Figure 1Q) at the stroma-DM interface up to 2 mm (Figure 1R). Air was injected to obtain a detachment of DM and stroma (Figure 1S) and the bubble was enlarged up to the corneal periphery (Figure 1T). If the bubble was not achieved then another PoI was considered and the bubble was enlarged from that site until a complete bubble was obtained. The air was removed by sucking it back in the syringe and the tissue was used for further analysis (Busin et al. 2010). The method is shown in supplementary video 5.

Time and cost analysis

The time for the preparation of graft was monitored by stopwatch and the costs were analyzed based on the most expensive instruments used in the technique.

Statistical analysis

Comparisons between these groups were performed by one-way ANOVA followed by Tukey test as a post hoc analysis test. Data were deemed significantly different when $p<0.05$.

RESULTS

Donor characteristics

Average age of the donors, gender, post mortem hours and endothelial cell density (ECD) from each group is listed in table 1. All the tissues were preserved in TCM not more than 28 days at 31^{O}C before the experiments.

Preparation time

The time required to prepare the grafts in all the groups was statistically significant ($p<0.05$) (Table 2). It was observed that M3 required longer graft preparation time compared to any other methods; M4 and M5 required the least time (Figure 2, Table 2). M1 and M2 required almost same time to prepare the graft.

Tissue wastage

There was no total tissue wastage from M1, M2 and M3. Two tissues were partially peeled (60%) from M1 (although not lost completely) due to tight attachment of the DM to stroma compared to others. M2 and M3 did not show any tissue wastage. One tissue was lost due to over-filling of stroma with liquid and not forming a liquid bubble in M4. Two tissues were lost due to stromal swelling caused by air, which did not generate a bubble in M5. There were three sites used for pneumatic dissection to generate air bubbles but all three failed in M5. The wasted tissues were not considered in this study and other tissues were obtained for evaluation purposes using the same technique.

Endothelial cell density, cell loss, cell death and morphology

Minimal cell death (assessed by trypan blue, Fig 3) was observed in M1 (Figure 3A), M2 (Figure 3B) and M3 (Figure 3C). Both M4 (Figure 3D) and M5 (Figure 3E) resulted in higher cell death than the other methods with M5 showing the highest percentage of dead cells (Figure 4, table 2). Cell death was statistically significant ($p<0.05$) between the groups (table 2). Endothelial cell loss was found to be highest in M5, however there was no significant difference between the groups (table 2).

The morphology in all the conditions did not differ pre- and post-preparation; however, there were some pleomorphic cells observed after Alizarin red staining (Figure 5A-5E). Denuded areas with no endothelial cells were noted after Alizarin red staining especially in M4 (Figure 5D) and M5 (Figure 5E).

Cost

Bubbling techniques (M4 and M5) were found to be least expensive with an approximate cost of $. <1.0 (majorly being the syringe) compared with the stripping methods that used an epithelial spatula ($. 8.0) or Sinskey hook ($. 10.0). The most expensive technique was M3 that used donor punches costing up to $. 150 also mentioned in table 3.

Hoechst, Ethidium Homodimer and Calcein-AM staining

Triple endothelial cell labeling with Hoechst 33342 (H), Ethidium Homodimer (E), and Calcein-AM (C) showed presence of 'E' in red representing the dead cells, blue representing the nuclei 'H' and green indicating the viable cells 'C'. Higher numbers of dead cells were observed in M4 (Figure 6D) and M5 (Figure 6E) [correlating with the data determined by trypan blue staining] compared with M1 (Figure 6A), M2 (Figure 6B) and M3 (Figure 6C). The number of apoptotic cells was very low and as the cells were compact it was difficult to evaluate the exact number of apoptotic cells in different conditions.

ZO-1 staining

Expression of ZO-1 was observed in all the conditions M1-M5 (Figure 7A-7E respectively) and the hexagonality was partially maintained in all the cases wherever the cells were present. The function of tight junctions was hence checked and the morphology was correlated with Alizarin red staining. There were small, denuded areas due to the detachment of endothelial cells where ZO-1 was not expressed.

DISCUSSION

DMEK has shown advantages such as early visual rehabilitation rates and outcomes. Endothelial keratoplasty using pre-cut for DSAEK or pre-stripped for DMEK grafts have shown increased interest recently and the eye banks are increasingly requested to produce these challenging grafts (Ruzza et al. 2015; Parekh et al. 2017a). The most suitable method for DMEK graft preparation has become an area for debate. Although protocols to standardize graft excision and transplantation have been introduced earlier (Dapena et al. 2011 and Groeneveld et al. 2013) however, as there are many procedures followed for DMEK, an affordable and easy to prepare a good quality graft must be identified, as this is one of the reasons why uptake of DMEK by surgeons is growing at a slower rate. Eye bank technicians have experience preparing a high volume of corneas for selective transplantation on a daily basis and therefore a pre-prepared graft from the eye bank could be a valuable option for the surgeons to reduce the graft preparation time, tissue wastage and overall costs in the surgery (Ruzza et al. 2015). Using pre-prepared tissue may, therefore, facilitate smoother surgery. As there are several possible techniques for DMEK graft preparation, we studied few methods that are routinely used and compared them to find out the most suitable technique for DMEK graft preparation. Hence, we selected three different ways of stripping and 2 bubble techniques and compared multiple parameters.

We observed that both, M4 and M5 are the techniques that use pressure between stroma and DM to create a bubble. Pneumatic dissection (M5) is carried out with full single force of air compared to SubHyS (M4) that utilized medium to high force of liquid depending on the primary small bubble. The pressure to obtain a complete bubble could have caused high cell death in M4 and M5 as it stretches the tissue. It was also observed that if the liquid is injected in the stroma, the force of the liquid cleaves the stroma and enters the stroma-DM plane to create a bubble. However, this phenomenon is not seen with air. The air is blocked in the stromal lamellae and as the density of air is less than liquid, it does not further cleave the posterior stroma and hence higher tissue wastage was noted in M5. Tissue wastage is significantly low in stripping techniques compared to bubble methods.

Time required to produce a bubble from M5 was least if the bubble is generated from the first PoI. M4 is a controlled method hence requires more time than M5. However, it depends on the tissue and technique, if the DM to the stroma is tightly attached, the liquid can leak from the PoI. In such cases, the tissue should be immediately switched to a stripping technique. M3 requires longer time for graft preparation due to an extensive technique that requires punching, separating the graft and peeling compared with relatively easy methods such as M1 and M2.

Stripping methods have shown reduced cell death rate. All three stripping methods with different PoIs were considered for this study. However, as M3 technique provides less endothelial damage, it could be worth investing the time but due to the expenses of this technique it may not be economically advantageous by many eye banks or surgeons with low volume grafts. Other techniques like M1 and M2 have also shown an acceptable range of cell death with both the methods having an economical advantage. The epithelial spatula or the Sinskey hook is not expensive and, therefore, if this technique is mastered, it can provide affordable and better quality DMEK grafts with minimal cell death.

For tightly attached tissues, a gentle oscillating stripping method can be used to strip the DMEK graft, however, for cases like previous cataract incision and horse shoe shaped tears, it is recommended that the PoI should be from the opposite ends of the incision or rejecting the area of incision and initiating the stripping just after the incision. A para-central DMEK graft can be achieved this way (Parekh et al. 2017b). However, these kinds of tissues can only be stripped and cannot be bubbled as the bubble will pop out and the tissue will be torn or the liquid will leak from the incision site never allowing a bubble generation. There are several graft excision techniques that have been published in literature with advantages and disadvantages as recently reviewed and reported by Birbal R. et al (Birbal R. et al 2017). The limitation of this study was that we were only able to get data on five techniques that are routinely prepared by our eye bank and collaborators.

As stripping techniques have provided better graft quality compared to bubble techniques in this study and also from our previous experience (Parekh et al. 2017b), it is recommended to use a stripping method. M3 has shown to have the best graft quality, however, as the overall expenses and time of preparation for this method is significantly high for any low volume eye bank, it may have strict limitations. Stripping techniques with epithelial spatula and Sinskey hooks have served the best in terms of graft quality and economic feasibility. From this study, we have observed that M2, tissue preparation using Sinskey hook, could serve as the best graft preparation method considering all the parameters that include cell death, endothelial cell loss, time required to prepare the graft and costs. We believe that the techniques described in this article would help the surgeons and eye bankers to select the best option for DMEK graft preparation.

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FIGURES

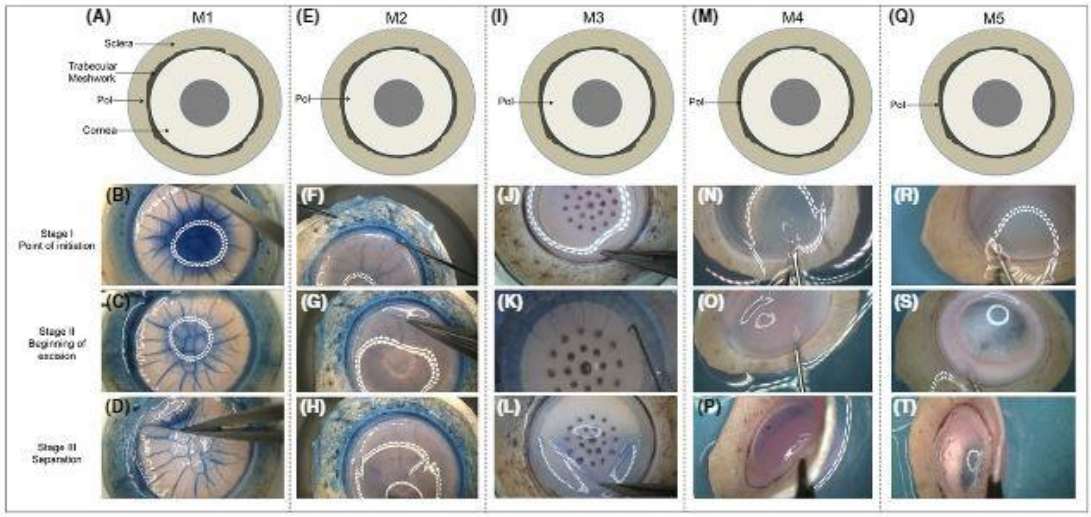

Figure 1: Schematic representation, stage I, II and III of different DMEK graft preparation methods.

M1 represents the technique that uses an epithelial spatula to swipe the DMEK graft all the way from the trabecular meshwork (TM). A) Point of Initiation (PoI) is shown just before the TM, B) PoI on a donor cornea, C) Excision begins after removing the TM at the periphery of the DMEK graft and D) Separation of a DMEK graft from the underlying stroma is carried out using acute forceps using stripping method.

M2 represents the technique that uses a Sinskey hook to score the peripheral endothelium. E) PoI is shown at the periphery of the endothelium just before the TM on the corneal side. F) PoI near the TM, the donor endothelium is scored using a Sinskey hook along the circumference of the cornea and G) Separated from the stroma followed by H) excision of DMEK graft using acute forceps using stripping method.

M3 represents the technique that uses a donor punch. I) PoI 9.5mm from the central cornea, J) Donor punch that is used for superficial tapping on the endothelial side, K) Cleavage hook used to separate the DMEK graft from the trephined site and L) Acute forceps used to strip and excise the DMEK graft from the cornea.

M4 represents the submerged hydro-separation technique. M) PoI is just beneath the TM, N) A needle of the syringe is advanced in the stroma-DM plane until the bevel is completely inside, O) with moderate to high pressure, the liquid is forced in the plane to create a liquid bubble, P) a full length liquid bubble separating the stroma with DM.

M5 represents the pneumatic dissection technique. Q) PoI is from the sclera just outside the TM, R) a 25G needle of the syringe is advanced tangentially, S) an air bubble is initiated with full force, if there is a central air bubble with stromal lamellae filled with air, the PoI can be changed to another site and the bubble is generated, T) A full length air bubble is thus created.

Note: The circle with white dots is an impression originating from the LED light fixed on the microscope.

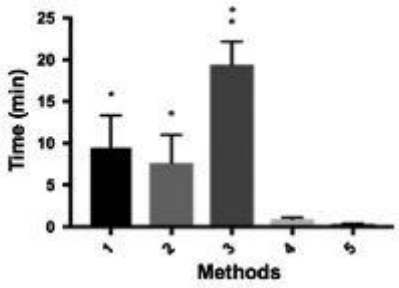

Figure 2: Time required to prepare the graft in each method was statistically significant with M3 taking highest time to prepare a graft. *Methods take more time than others. **Method is the slowest of all.

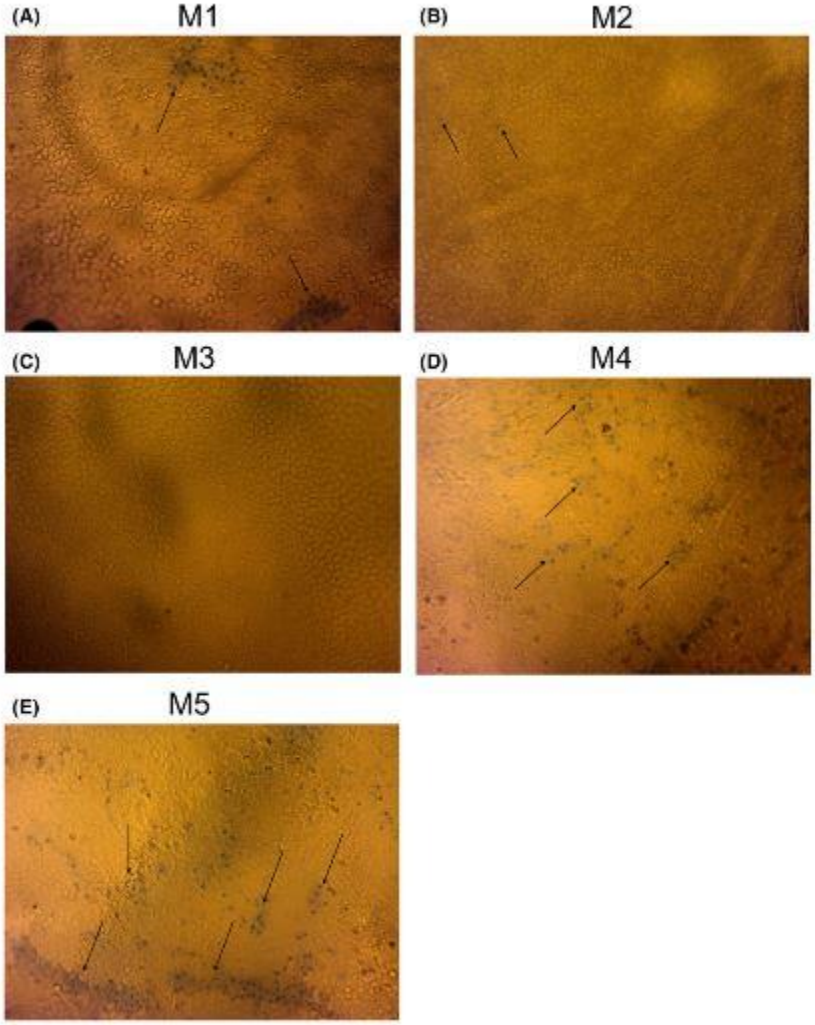

Figure 3: Endothelial cell density and cell death observed using trypan blue positive cells (marked with arrow). A) M1 showed acceptable range of cell death, B) M2 showed minimal amount of cell death, C) M3 did not show any cell death, D) M4 and E) M5 showed critical levels of cell death.

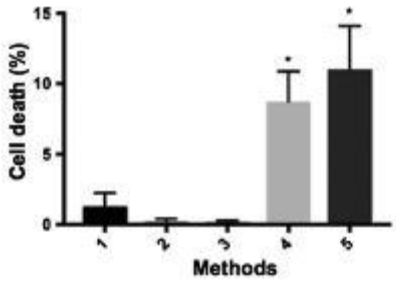

Figure 4: Endothelial cell death was statistically significant ($p<0.05$) between all the methods. *Methods cause significant cell death compared to others.

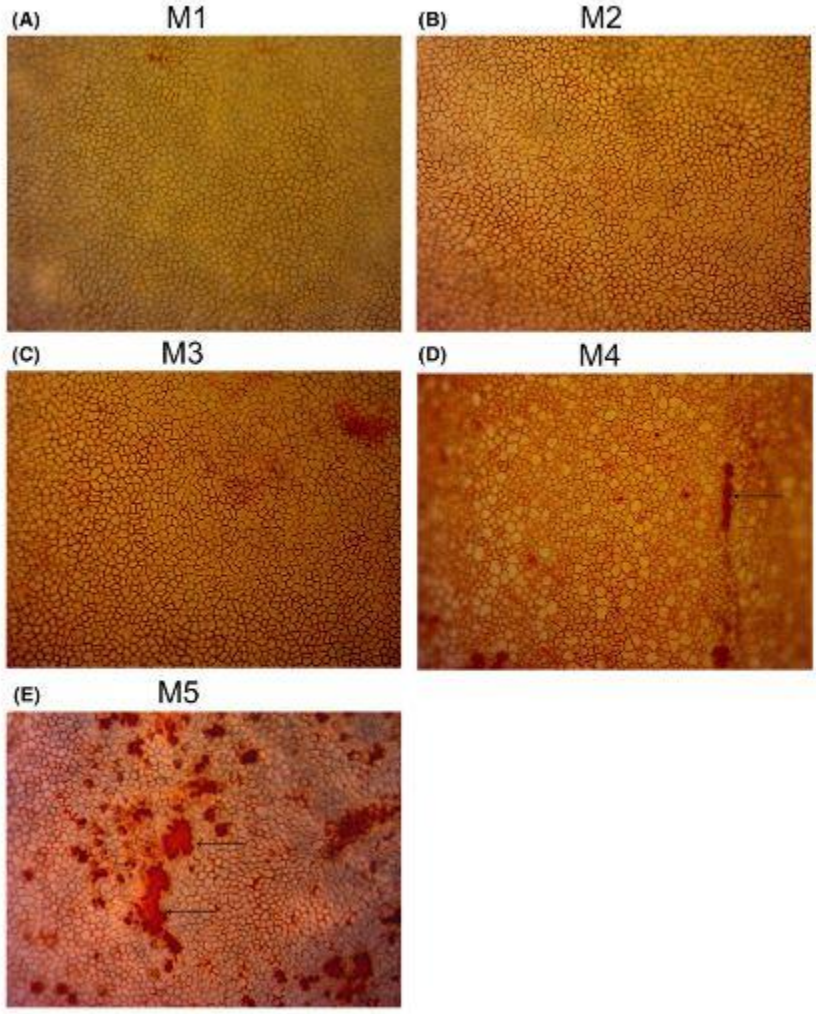

Figure 5: Alizarin red staining for morphological analysis showed A) M1, B) M2 and C) M3 that did not differ in morphology however, D) M4 showed pleomorphic cells and areas without cells (marked with arrow) and E) M5 showed pleomorphic cells and severe amount of denuded areas (marked with arrows). Scale: 100X magnification.

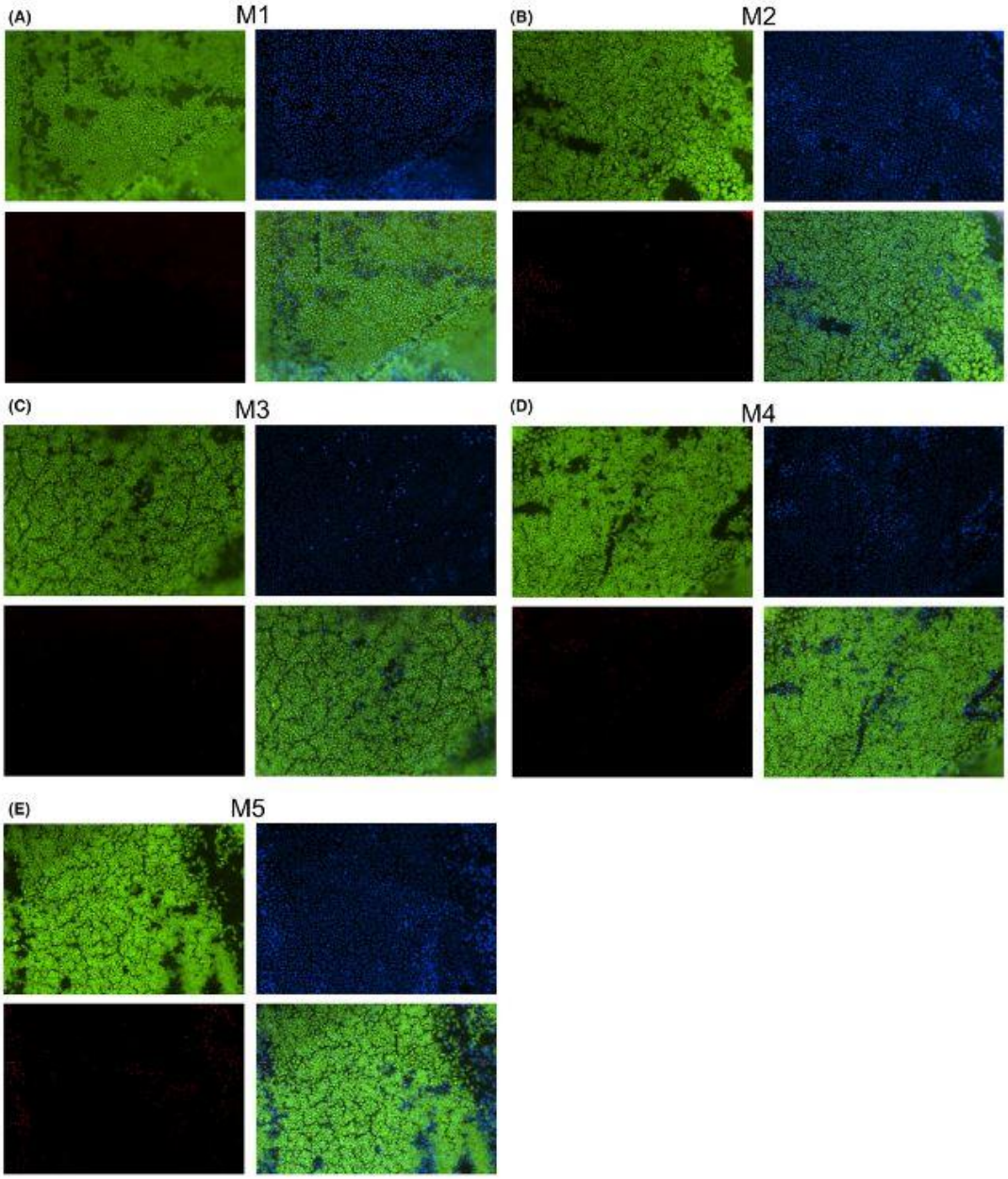

Figure 6: Hoechst, Ethidium homodimer and Calcein AM (HEC) staining to determine live/dead cells. Live cells were determined by green (Calcein AM staining), nuclei of the cells were determined by blue (Hoechst staining) and the dead cells were determined with red (Ethidium homodimer staining) color. All the results were correlated with trypan blue staining. It was observed that A) M1 and B) M2 showed acceptable cell death but C) M3 did not show any cell death whereas D) M4 and E) M5 showed critical levels of dead cells. There were some denuded areas also found in M4 and M5.

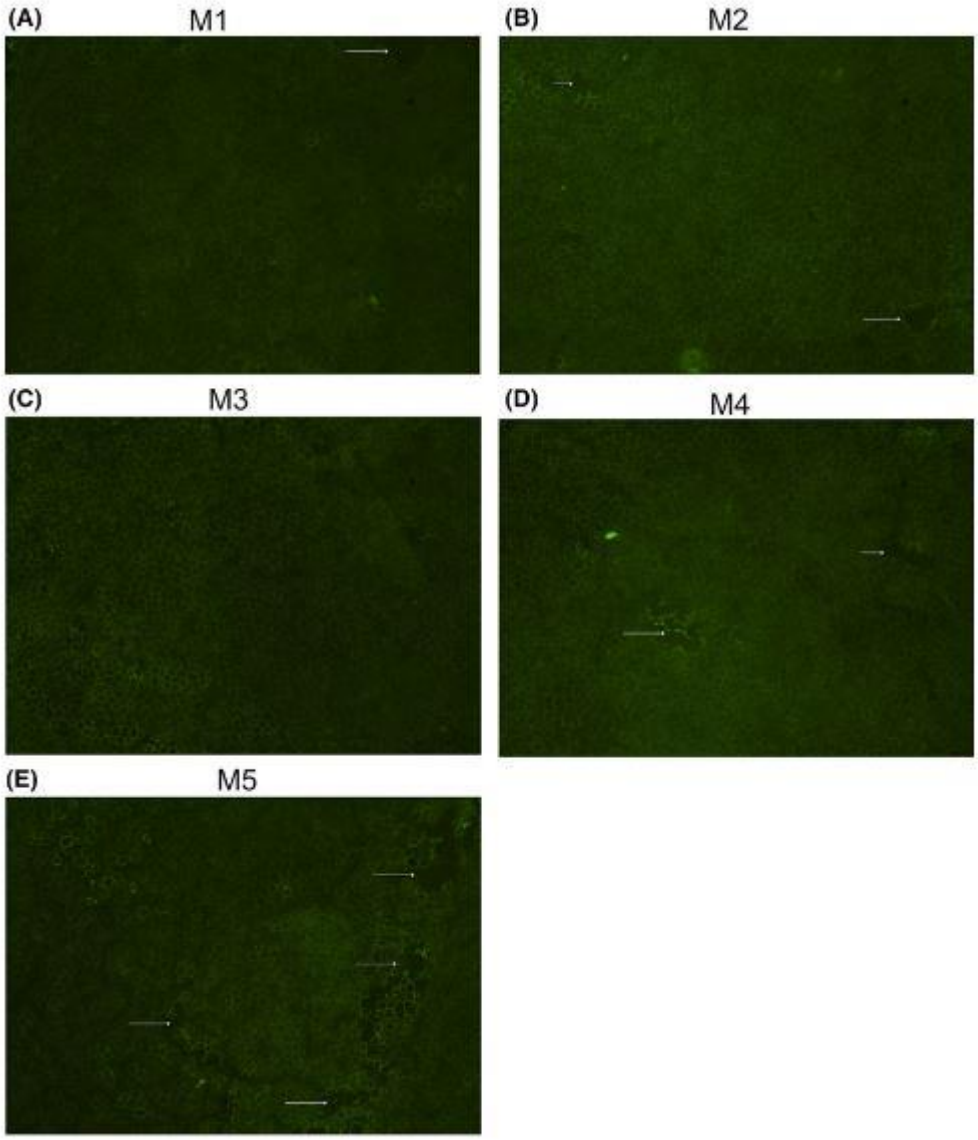

Figure 7: Zonula Occludens-1 expression was seen in all the methods wherever the cells were present. A-E) There were denuded areas as observed earlier and ZO-1 was not expressed in those areas. The morphology and hexagonality was observed and normal to pleomorphic cells in all the cases were found. Scale: 100X magnification.

TABLES

	Age (Years)	Male/Female	Post mortem (Hours)	ECD (cells/mm^2)
M1	75.0(3.3)	6/1	16.8(5.3)	2042(113.3)
M2	73.3(2.5)	4/3	12.5(4.1)	1971(48.8)
M3	73.0(4.1)	5/2	10.7(6.9)	1975(95.7)
M4	65.8(10.4)	5/2	13.7(6.3)	1960(54.8)
M5	72.1(3.9)	4/3	10.1(3.6)	2042(78.7)

Table 1: Donor characteristics in terms of age, gender, post mortem hours and endothelial cell density from corneas used in each methods M1-M5. All the data are presented as Mean(SD) except the data on gender. ECD stands for Endothelial Cell Density.

	Dead cells (%) Mean (SD) [Range]	ECD (cells/mm^2) Mean (SD) [Range]	ECL (%) Mean (SD) [Range]	Time (minutes) Mean (SD) [Range]
M1	1.29(0.96) [0.3-3]	1928(75) [1800-2000]	2.71(4.99) [0-10]	9.43(3.91) [4.5-15]
M2	0.21(0.17) [0-0.5]	1921(90) [1800-2000]	2.96(7.42) [0-14.2]	7.64(3.37) [4.5-14]
M3	0.13(0.14) [0-0.4]	1957(97) [1800-2100]	1.18(7.37) [0-4.76]	19.41(2.77) [15-23.5]
M4	8.71(2.14) [8-12]	1914(89) [1800-2000]	3.34(7.25) [0-10]	0.27(0.14) [0.1-0.4]
M5	11.00(3.11) [8-16]	1900(81) [1800-2000]	4.06(7.06) [5-10]	0.17(0.16) [0.7-0.2]
pValue	**<0.05***	0.96	0.96	**<0.05***

Table 2: Comparison between different graft preparation methods: 1° column cell death evaluated with trypan blue, 2° column ECD evaluated with light microscope, 3° column overall endothelial cell loss, 4° column time evaluated with stopwatch for each method.
Key: ECD – Endothelial Cell density; ECL – Endothelial Cell Loss; *Statistically significant values

	Instrument	Diameter of graft obtained (mm)	Risk of peripheral tears	Risk of bubble burst	Suction base	Forceps*	Costs** Approx (USD)
M1	Epithelial Spatula	11	High	Not applicable	Not mandatory	Medium forceps (J3683, e.Janach) size 3, straight, pointed tips, 120 mm	8
M2	Sinskey Hook	10.5	High	Not applicable	Not mandatory	Medium forceps (J3683, e.Janach) size 3, straight, pointed tips, 120 mm	10
M3	Donor Trephine	8.25-10 (Wide range)	Moderate	Not applicable	Mandatory	Medium forceps (J3683, e.Janach) size 3, straight, pointed tips, 120 mm	150.0
M4	25G needle with 1 mL syringe	11	Not applicable	Moderate	Not required	Not applicable	<1
M5	25G needle with 1 mL syringe	11	Not applicable	Moderate-High	Not required	Not applicable	<1

Table 3: Parameters considered: instruments used, diameter of the graft, risk of peripheral tears or bubble burst, inclusion of suction base, forceps and overall costs.
*Forceps – depends on the surgeon. We prefer acute forceps as they grasp sufficient tissue required for peeling and to reduce overall endothelial cell damage.
**Costs – the overall costs are mainly for the instruments that are used. As these costs are the primary costs that an eye bank or the surgeon will have to pay, we considered this as our point of reference. This mainly includes the price of the instrument (Column one).

SUPPLEMENTARY VIDEO LEGENDS

Supplementary video 1: Stripping from the trabecular meshwork – Epithelial spatula was used to swipe the trabecular meshwork (TM) towards the cornea to release the peripheral endothelium. The TM was cut and using trypan blue as visualizing dye, the Descemet membrane endothelial keratoplasty (DMEK) tissue was peeled.

Supplementary video 2: Stripping by scoring the peripheral endothelium – Sinskey hook was used to score the peripheral endothelium and the DMEK was peeled from the scored area.

Supplementary video 3: Stripping by punch method – A 9.5mm donor punch was used to superficially trephine the endothelium. Cleavage hook was used to identify the cleavage plane followed by stripping the tissue.

Supplementary video 4: Submerged Hydro-Separation (SubHyS) Method – 25G needle attached to a 1 mL syringe filled with tissue culture medium was injected between the stroma and Descemet membrane. Medium to high pressure was used to create a liquid bubble.

Supplementary video 5: Pneumatic dissection method – 25G needle attached to 1 mL syringe filled with air was injected between the stroma and Descemet membrane. Full pressure was applied to create an air bubble.